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The Anti-Inflammatory Activity of Humic Acid from Borneo Peat Soil in Mice

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ABSTRACT

Humic acid is a humus compound found in peat soil. Humic acid can potentially be used as an anti-inflammatory compound. This study aimed to determine the effect of humic acid on the volume of foot mice edema and to find the best dose that can suppress the degree of edema volume. The animal object was Swiss mice weighing 25-30 grams and 3 months old. The study used Randomized Block Design (RBD) with positive control, negative control and humic acid treatment with dose 62.5 mg kg^{-1} BW, 125 mg kg^{-1} BW, and 250 mg kg^{-1} BW. The result of this research showed that edema inhibition by the administration of humic acid dose 62,5; 125; 250 mg kg^{-1} had inflammatory inhibition percentage 2.67%, 13.34%, and 20.01% respectively in 5-hour observation. The best dose of humic acid to suppress inflammation in the mice's paw is a humic acid dose of 250 mg kg^{-1} compared with value 23.3% of sodium diclofenac as the positive control.
Keywords: Humic Acid; Peat; Edema; Carrageenan

INTRODUCTION

Peat soil is a type of soil found in Indonesia. The amount of peatland in Indonesia is ranked as the 4th largest in the world with 14,905,574 hectares, which spreads across the islands of Kalimantan, Sumatra, and Papua. Kalimantan has the second-largest amount of peatland after Sumatra with an area of 4,778,004 hectares (Agricultural Research and Development Agency, 2011).

Approximately 50% to 75% of the total peat soil contains organic soil matter and approximately 75% of this organic soil matter consists of humus compounds (Tan, 1995). Humus has a chemical composition, structure and functional groups that can change depending on the starting material, age and environmental conditions during the humification process (Kodama *et al*., 2007). Based on the solubility of the compound in water, humus is divided into humic acid, fulvic acid and himatomelanic acid (Schepectin *et al*., 2003). The duration of decomposition affects the content of peat soil humus. Humic acid will increase while the content of fulvic acid and himatomelanic acid decreases. Therefore the use of humic acid will be more effective on peat that has decomposed at the proper rate, which is sapric peat (Agustian *et al*., 2004).

Humic acid can potentially be used as an antibacterial, antiulcerogenic, antiallergic and antitoxic (Schnepectin *et al.,* 2002). Kodama and Denso have done *in vitro* studies (2007) using a concentration of 3% and 6% humic acids of subtropical humus on mice to suppress cell growth L1210. This slows the formation of the tumor mass cells by 170 cm^3 and 100 cm^3 within 12 days after the injection of the tumor cells. Rousdy & Wijayanti (2016) and Rousdy *et al*. (2016) reported humic acid from Borneo peat soil has triggered the total leucocytes count in mice and carp fish. Rousdy & Wardoyo (2018) also reported the effects of humic acid in stimulating the immune system through increasing white pulp and germinal center diameter of the spleen.

In addition to stimulating the immune system, humic acid has the effect of suppressing the immune system, depending on the dose given. Research by Junek *et al*. (2009) stated that the administration of humic acid from subtropical Germanic Alteich peat caused a dose-dependent bimodal effect on the release of TNF-α cytokines in cell culture. Low concentration of humic acid (10-80 mg ml^{-1}) increases the release of TNF-α whereas high concentration humic acid (4100 mg ml^{-1}) reduces the release of TNF-α. This aspect is supported by Constance *et al.* (2009) reported that humic acid from subtropical peat *in vitro* acts as an anti-inflammatory by suppressing the formation of interleukin-1 (IL1), interleukin-6 (IL6) and *tumor necrosis factor alpa* (TNF-α) which act as a proinflammatory cytokine.

Antiinflammation is the ability of a compound to suppress swelling and agitation that occurs due to introducing foreign substances in animals. Inflammation is an irritation that occurs in body tissues characterized by heat, increased edema (swelling), redness and pain (Ricciotti &

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Fitzgerald, 2011). The acute inflammatory test model used in this study is carrageenan induction. Introducing carrageenan can trigger the release of prostaglandins which are inflammatory hormones, causing swelling in test animals (Vinegar *et al*., 1976).

Peat is also widely found on the island of Borneo. One of the peat's contents is humic acid. Humic acid is an anti-inflammatory compound. A study on the rate of inflammation in mice as a result of introducing carrageenan needs to be conducted.

METHODOLOGY

Materials

This research was conducted in December until February 2017. The research was conducted in Laboratory of Zoology and Biology Laboratory, Faculty of Mathematics and Natural Sciences University of Tanjungpura Pontianak. The research used Randomized Block Design (RBD) with five treatments namely the negative control, the positive control using sodium diclofenac solution and humic acid dose 62.5; 125; 250 mg kg^{-1} BW. All treatment replicated five times.

Peat Soil Sampling and Separation of Humic Acid

Peat soil was taken in a depth of 80 cm from the surface. Type of peat was analyzed in the Soil Laboratory, Faculty of Agriculture, Tanjungpura University, to determine the maturity level of peat soil. The peat soil that has been taken is cleaned from the roots, twigs, and leaves of plants. Then the peat soil was dried for 3 days and mashed with mortar. The separation or extraction of humic acid peat soil refers to IHSS method (2012). The separation process based on precipitation in the strong acid and solubility in the weak base.

Preparation of Testing Animal

The testing animals used in this study were 25 Swiss male mice (*Mus musculus*) with weight ranging from 25-35 grams and 3 months age. The mice collected from animal breeders in Pontianak. The mice were acclimated for 7 days using a cage at room temperature 26-32^{o}C. Each cage consists of five mice. During acclimation, the mice were fed and drunk sufficiently.

Anti-inflammatory Test Procedure

Antiinflammatory procedure refers to Winter *et al*., (2002) obtained based on the preliminary test. The carrageenan lambda powder was diluted with NaCl 0.9%. The determination of 0.15 ml of a 1% carrageenan suspension used in the study was based on the Winter method modified through the preliminary test. The preliminary test In the preliminary test, 1% carrageenan injection of 0.15 mL gave the largest volume of edema compared to 0.1 mL and 0.2 mL doses.

Carrageenan induction was performed by injecting 0.15 ml of 1% carrageenan suspension at the subplantar foot of the mice. The edema volume formed on mice's paw was measured using a plethysmometer, based on Archimedes' law (Winter *et al*., 2002). Before induction, the left leg marked on the ankle then the mice's paw volume measured with plethysmometer. Mice were given humic acid and standard drug sodium diclofenac in 30 minutes before induced with carrageenan. After 30 minutes, the subplantar foot was injected with 0.15 ml carrageenan 1%. The paw volume of mice was measured at 0, 1, 2, 3, 4, and 5 hours after induced. The edema volume was represented the deficiency of initial paw volume (Vi) and paw volume after induction of carrageenan (Vt) (Eq.1). They are under the curve (AUC) was calculated by (Eq.2). The percentage of edema inhibition was calculated using AUC value (Eq.3) by the formula (Sahlan *et al*., 2019):

$$V_e = V_t - V_i \quad (1)$$

$$\text{AUC} = \frac{Ven+Ve(n-1)}{2} x\ (tn - t(n-1 \quad (2)$$

$$\text{Inflammatory Inhibition (\%)} = 1 - \left(\frac{AUCt}{AUCnc}\right) x\ 100\% \quad (3)$$

Statistical Analysis

The data obtained were analyzed by the One-Way Analysis of Variance (ANOVA) test with 95% confidence interval. If there is a significant difference between treatment, it was analyzed by Duncan Multiple Range Test (Besral, 2010). Data analysis was done with SPSS program version 16.

RESULT AND DISCUSSION

After induction of 1% lambda carrageenan, all treatment groups showed edema in paw (Table 1, Figure 1). The peak of edema volume occurred in 3-4 hours after induction but each treatment group showed a different decreasing volume. The negative control treatment gives the highest edema volume 20,8 mm^3 in 4 hours. This occurred because in negative control there was not an active compound to inhibit the inflammatory response. The lowest dose of humic acid 62,5 mg kg^{-1} also gives high edema volume 18,9 mm^3. This also occurred because a lower dose of humic acid is not enough to inhibit the inflammatory response.

The lowest degree of edema occurs in a positive control (sodium diclofenac) treatment. In sodium diclofenac, the edema volume at the 5th hour is 12.8 mm^3. The edema volume of sodium

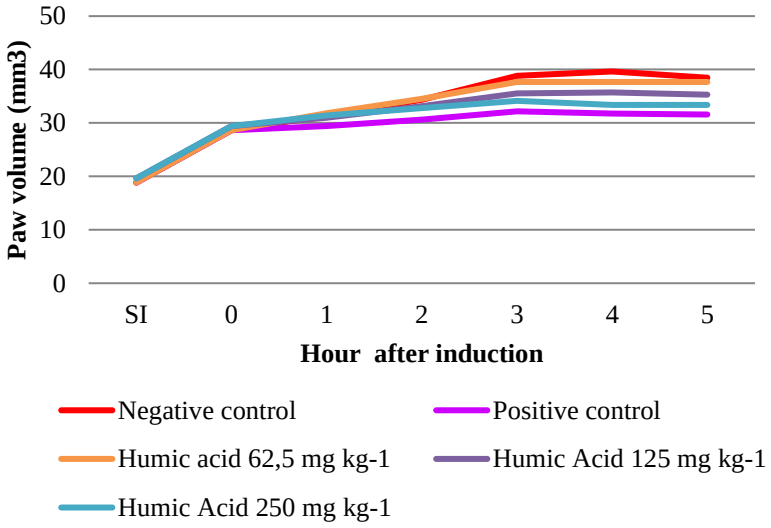

Figure 1. Average paw volume (mm^3)

Table I. Edema volume of paw mice after carrageenan induction

Test groups	Average edema volume (mL)					
	Ve_0	Ve_1	Ve_2	Ve_3	Ve_4	Ve_5
Negative control	9.8±1.7^a	12.6±2.7^b	15.5±1.7^b	20.0±2.9^b	20.8±2.1^c	19.7±1.7^c
Positive control	9.8±1.7^a	10.6±2.7^a	11.8±2.9^a	13.4±2.9^a	13.0±2.9^a	12.8±2.6^a
Humic acid 62.5 mg kg^{-1}	9.8±1.7^a	13.0±2.1^b	15.7±1.1^b	18.9±2.1^b	18.9±2.1^c	18.9±2.1^c
Humic acid 125 mg kg^{-1}	9.8±2.7^a	11.4±3.7^{ab}	13.6±2.2^a	15.9±2.6^a	16.1±4.0^b	15.7±4.1^b
Humic acid 250 mg kg^{-1}	9.8±2.7^a	12.6±3.1^{ab}	13.2±2.4^a	14.5±1.0^a	13.8±2.4^a	13.8±2.4^{ab}

Note: Ve_0 = edema paw volume in 0 h. Ve_1 = edema paw volume in 1 h. Ve_2 = edema paw volume in 2 h. Ve_3 = edema paw volume in 3 h. Ve_4 = edema paw volume in 4 h. Ve_5 = edema paw volume in 5 h. Data are average±standart deviasi. Different value in the same column showed significant difference (P<0,05)

diclofenac treatment was not significantly different from humic acid 250 mg kg^{-1} (Table I). It means humic acid dose 250 mg kg^{-1} has potential anti-inflammatory substances.

The area under the curve (AUC) can provide information about the potential of humic acid in reducing inflammation. The greater the AUC value means the smaller effect of decreasing edema volume and the smaller the AUC value means the greater the effect of decreasing edema volume (Apridamayanti *et al*., 2018). At 1st hour, the AUC values of all test groups were not significantly different (P>0,05). The inflammatory response showed a different pattern at the 2nd to 5th hour and significantly different between the test group (Table II).

The negative control had the highest AUC value (83,61). In contrast with the positive control of sodium, diclofenac has the lowest AUC value (59,97). AUC value of humic acid dose 250 mg kg^{-1} also not significantly different (P<0,05) with the positive control (64,96). However, the AUC value of humic acid dose 62 mg kg^{-1} was not significantly different from the negative control, showed a low inflammatory inhibition.

The AUC value inversely with the inflammatory inhibition percentage. The positive control which had the lowest AUC value showed the highest inflammatory inhibition percentage (Table 3). The humic acid dose of 62.5 mg kg^{-1} was significantly different (P<0.05) with humic acid dose of 125 and 250 mg kg^{-1}. It shows that the anti-inflammatory effect of humic acid doses of 62.5 mg kg^{-1} is lower than the dose of humic acid 125 and 250 mg kg^{-1}. Meanwhile, the humic acid dose 250 mg kg^{-1} creates the most anti-inflammatory effect and almost equal with the positive control (diclofenac sodium).

Anti-inflammation is a test that aims to determine the ability of drugs to suppress edema. Edema is a result of the introduction of foreign substances into the body. A standard model of testing inflammation is the introduction of carrageenan into the hind legs of mice

Table II. Area under the curve (AUC) value per each test groups

Test groups	AUC (mL.hour)					
	1	2	3	4	5	Total
Negative control	11.19±0.8^{a}	14.03±0.9^{b}	17.76±0.9^{c}	20.41±0.8^{b}	20.22±0.7^{c}	83.61±0.0^{c}
Positive control	10.21±0.8^{a}	11.19±1.5^{a}	12.56±1.5^{a}	13.15±1.1^{a}	12.86±1.3^{a}	59.97±3.5^{a}
Humic acid 62.5 mg kg^{-1}	11.39±1.2^{a}	14.33±1.5^{b}	17.27±1.5^{c}	18.84±1.7^{b}	18.84±1.6^{c}	80.67±4.4^{c}
Humic acid 125 mg kg^{-1}	10.60±1.9^{a}	12.46±1.6^{ab}	14.72±1.8^{b}	15.99±2.1^{a}	15.89±1.6^{b}	69.67±6.0^{b}
Humic acid 250 mg kg^{-1}	10.79±0.9^{a}	12.46±1.6^{a}	13.84±1.7^{ab}	14.13±2.2^{a}	13.74±1.4^{ab}	64.96±3.5^{ab}

Note: Ve_0 = edema paw volume in 0 h. Ve_1 = edema paw volume in 1 h. Ve_2 = edema paw volume in 2 h. Ve_3 = edema paw volume in 3 h. Ve_4 = edema paw volume in 4 h. Ve_5 = edema paw volume in 5 h. Different value in the same column showed significant difference (P<0,05)

Table III. Percentage of edema inhibition

Test groups	Inflammatory Inhibition (%)
Positive control	23.33^{a}
Humic acid 62.5 mg kg^{-1}	2.67^{c}
Humic acid 125 mg kg^{-1}	13.34^{b}
Humic acid 250 mg kg^{-1}	20.01^{ab}

(Chakraborty *et al*., 2004). Carrageenan is a derivative of polysaccharides that the body will determine is a foreign substance and thus cause swelling. Carrageenan causes phospholipids in cell membranes to damage and produces arachidonic acid with the help of a phospholipase enzyme. Subsequently, the arachidonic acid will enter the lipooxygenase and cyclooxygenase pathways to form inflammatory mediators (leukotrienes, prostaglandins, and thromboxanes).

Carrageenan induction is then carried out subplantarly on the soles of the mice. Subplantar carrageenan induction will increase levels of cyclooxygenase-2 (COX-2) which will be inhibited by the anti-inflammatory drug (Ricciotti & Fitzgerald, 2011; Turnbach *et al*., 2002). Based on the results, the amount of swelling in each treatment continues to grow from 30 minutes to 300. The highest peak of the edema occurs in the minute 210 and tends to plateau in the next minute. Vinegar *et al.* (1976) reported that after 180 minutes of carrageenan induction there will be the second phase of inflammation characterized by prostaglandin release and edema will develop rapidly until 300 minutes.

Antiinflammatory activity is associated with inhibition of inflammatory mediator formation. Non-steroidal anti-inflammatory drugs (AINS) used in this study are diclofenac sodium can inhibit the formation of prostaglandins, thromboxane, and prostacyclin through inhibition of cyclooxygenase enzymes so that arachidonic acid is not converted into inflammatory mediators. Diclofenac sodium is also thought to suppress the migration of inflammatory leukocytes of the leukocytes, thereby reducing the formation of inflammatory mediators (Ammar *et al*., 2005).

Humic acid is known to have anti-inflammatory capabilities because it can increase lymphocyte proliferation and increase the production of interleukin 2 (Il-2) which serves to stimulate the development of natural killer cells and synthesize antibodies (Joone *et al*., 2003). According to Rousdy *et al*., (2016) the administration of humic acid depends on the dose, at low doses humic acid tends to increase the number of neutrophils and stimulate edema so that the resulting anti-inflammatory effect is smaller at low doses. However, humic acid dose 250 mg kg^{-1} BW increases the number of lymphocytes and decreases the number of neutrophils and monocytes so it gives the anti-inflammatory process.

Humic acid can inhibit the complement receptor 3 (CR3) neutrophils to bind with intracellular cell adhesion molecule-1 (ICAM-1) or vascular endothelial adhesion molecule-1 (VCAM-1) so that neutrophils cannot attach and marginate on blood vessel walls (Joone *et al*., 2004). According to Gau *et al*., (2000) humic acid can

inhibit the expression of ICAM-1 molecules), VCAM-1 and E-selectin in blood vessel endothelium so that it can inhibit leukocytes to stick on the walls of blood vessels.

The mechanism of the inflammatory response is a process involving leukocytes, especially neutrophils and monocytes. The entry of leukocytes into the tissues is mediated by chemical signals through the chemistry and adhesion molecules produced at the vascular endothelium and cells in the inflamed tissue. Endothelial vascular adhesion molecules that play a role in neutrophil marginations are ICAM-1 (intracellular cell adhesion molecule 1), and VCAM-1 (vascular cell adhesion molecule 1). Other proteins in endothelial adhesion are E-selectin and P-selectin. Both endothelial proteins are tasked to interact with leukocyte ligands to mediate while leucocyte margination in vascular endothelium (Klaus, 2003).

The ICAM-1 and VCAM-1 molecules will be generated by the endothelial when the inflammatory process occurs. Expression of ICAM-1 and VCAM-1 is influenced by the cytokines produced by monocytes and lymphocytes. When Tumor Necrosis Factor Alpa (TNF-α) and Interleukin-1 are produced, ICAM-1 and VCAM-1 are expressed so that the leukocytes can migrate into the inflammatory tissues. According to Zapolzka and Naruszewicz (2009), inhibition of TNF-α and Il-1 cytokines can inhibit the expression of ICAM-1 and VCAM-1 to suppress leukocyte migration into inflammatory tissues.

Humic acid can be anti-inflammatory because it can inhibit the formation of proinflammatory cytokines (Il-1, Il-6, and TNF-α) (Constance *et al*., 2009). Cytokines play an important role in the anti-inflammatory process. Cytokines that play a role in the anti-inflammatory process are interleukin1 (Il-1), Interleukin 2 (Il-2), Interleukin 4 (Il-4), Interleukin 6 (Il-6), Interleukin 8 (Il-8), Interleukin 10 -10), TNF-α, and gamma interferon. Interleukin 1 (Il-1) acts as a macrophage activator in the inflammatory process and synthesizes interleukin 6 (Il-6). Interleukin 2 (Il-2) serves to stimulate the growth of NK (natural killer) cells. Interleukin 6 (Il-6) is produced by macrophages, endothelial cells, and T cells. Interleukin 6 (Il-6) plays an important role in the acute inflammatory process and becomes a T cell stimulator. Interleukin 8 (Il-8) is produced by endothelial cells and the role of initiating the inflammatory process. Interleukin 10 (Il-10) produced by macrophages acts as a regulator of immunostimulant effects in various cells (Rao, 2005, Mark and Saunders, 2006)

TNF-α cytokines are produced by macrophages, NK cells, and mast cells. TNF-α plays an important role as an inflammatory mediator, at low doses can decrease adhesion molecule expression in endothelial cells, neutrophil macrophages, and lymphocytes. High levels of TNF-α can damage cells (Rao, 2005; Mark and Saunders, 2006). Thus the antiinflammatory capability of humic acid is suspected by inhibiting the production of proinflammatory cytokines.

Oral administration of the humic acid enters the digestive tract of the testing animal (Visser, 1973 *in* Johan, 2000). Then the humic acid will enter the blood circulation and will be metabolized in hepar. Based on the Johan (2000) research, potassium humic administration with a dose of 60 mg kg^{-1} BW, given orally 60 minutes before carrageenan induction can trigger inhibition of edema in 240 minutes. This suggests that humic acid is metabolized rapidly by the body after being administered orally.

The humic substance is polyanionic so it is easy to bind with organic matter and can reduce the effects of inflammatory mediators. The humic structure has consisted of a negatively charged ion (polyanionic) as an example hydroxyl or carboxyl group. The presence of negative ion within the functional group may cause anti-inflammatory effects by affecting the production of cytokines so that IL-1, TNF-α, and IL-6 may be inhibited.

CONCLUSION

Oral administration of peat soil humic acid can be act as an anti-inflammatory agent on the foot of mice that have been induced with carrageenan. Humic acid dose 62,5; 125; 250 mg kg^{-1} had inflammatory inhibition percentage 2.67%, 13.34% and 20.01% respectively. Humic acid dose 250 mg kg^{-1} BW gives the best response to suppress inflammation in the paw of mice induced by carrageenan.

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